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13. ABSTRACT (Maximum 200 Words)

Successful delivery of the HER-2/neu derived p369 peptide vaccine, KIFGSLAFL, to a large population of skin Langerhans cells (LC) by transdermal electroporationis expected to enhance the CTL response to breast cancer cells. Strategies have been developed to improve the electroporation protocol to enable the delivery of peptides. By using anionic lipids and detergents, the upper molecular weight limit of transdermal delivery by electroporation has been extended to beyond 10,000. This enables the delivery of antigenic peptides but not minigenes. With the new design of skin electrode, the transdermal flux of antigenic peptides was measured to be in the order of 10 μg/cm^2/min. Electric pulses also stimulates the migrationof skin Langerhans cells. Antigen-specific CTL response in immunized mice was measured by ELISPOT of gamma-interferon production. Earlier attempts to generate CTL response to KIFGSLAFL by electroporation delivery were negative, due to weak immune response. CTL response to electroporation delivery of the peptide vaccine SIINFEKL was positive, although it was not as effective as intradermal injection. We attribute the weaker response to the necessity to inject adjuvant separately. Effort to simultaneously deliver lower molecular weight CpG-containing oligonucleotide adjuvant by electroporation is underway. Delivery of p369 HER-2/neu derived peptide vaccines will be re-examined.

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Transdermal electroporation, vaccine, HR-2/neu peptide antigen

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INTRODUCTION:

In order to enhance the immune response against breast cancer, we proposed to introduce the HLA-A2 binding p369 peptide, KIFGSLAFL, derived from breast cancer associated antigen HER-2/neu, to the dermal Langerhans cells (LC), to stimulate the cytotoxic T-lymphocyte (CTL) response to breast cancer cells. The peptide or the minigen encoding it are to be transported across the skin by transdermal electroporation, which can cover a large skin area. We proposed to optimize the electric parameters for maximum delivery, to monitor both the migration of LC after exposure, and the resulting CTL response to the peptide antigen.

BODY:

1. <u>Enhancing the molecular transport and extending the molecular weight limit of transdermal electroporation delivery technology</u>:

A major problem facing the transdermal delivery of vaccine by electroporation is that the quantity of vaccine transported by electroporation may be limited, and that there is an upper limit of molecular size of the vaccine and adjuvant that can be transported through the skin without injection. The efficiency of transdermal delivery by electroporation was measured using FITC-labeled Dextrans of different molecular weights. Excised porcine epidermis or murine skin was placed in between two chambers of a Vertical Diffusion Holder (figure. 1). We found that those charged molecules with molecular weight less than 1,000 could be transported through the skin efficiently. The flux was in the order of 1-10 $\mu g/cm^2/min$ when 1 msec pulses of 100V were applied to the skin at 1 Hz. This is similar to the flux we measured in live mice (Johnson et al., 1998, 2002). Molecules at a higher molecular weight were transported at a lower flux, which dropped off significantly when molecular weight exceeded 1,000 (Sen et al., 2002). Applying anionic lipids to the skin during pulse application enhanced the transport flux and extended the molecular weight limit for efficient transport to beyond 4,000 (figure 2). However, transport of molecules with molecular weights at and beyond 10,000 still posted difficulty. This finding agreed with those reported by Lombry et al., (2000). With this finding, transdermal transport of minigenes would be a problem. We had to restrict the delivery to that of antigenic peptides alone (MW~9,000).
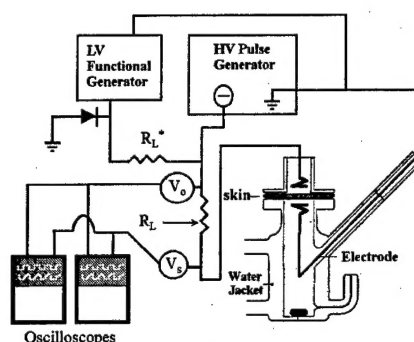

Figure 1. Schematic drawing of the Vertical Diffusion Holder and electrode arrangement.
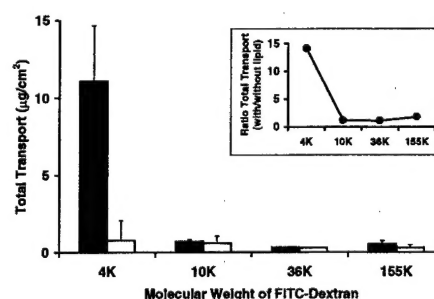

Figure 2. Transport of FITC-Dextrans of different molecular weights after 1 min of electroporation (100V, 1ms pulse width at 1Hz) with (■) and without (□) anionic lipids. Inset: Plot of the enhancement ratio of the total transport with and without added lipid.

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We found that transdermal transport increases non-linearly with temperature. Of particular significance is that there is a transition at 37-40°C that the transport increases by several folds within a 3 degree range. This is important for less efficient transport of macromolecules such as peptides and Dextrans of molecular weights 4,000 to 10,000. In addition, the kinetics of transdermal transport during and
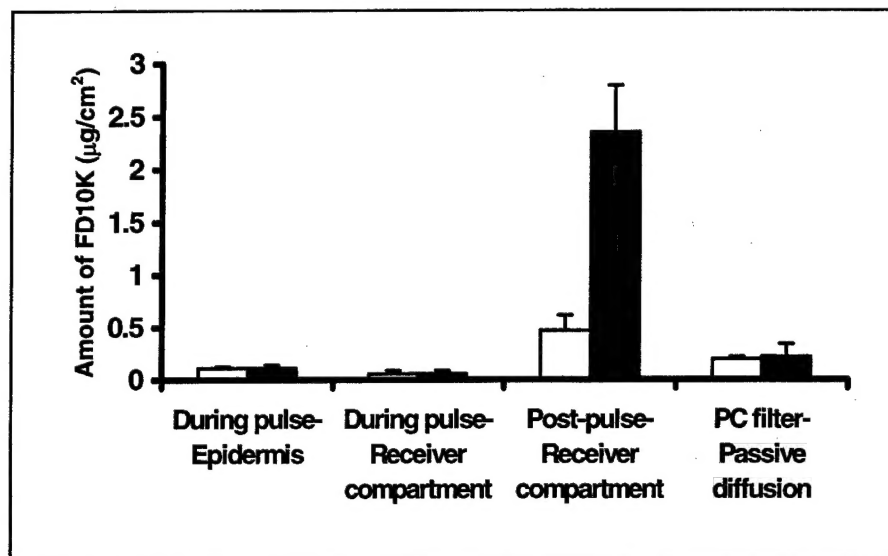

Figure 3. Total transport of FTIC-labeled Dextran 10kD (FD10k) into and through porcine epidermis either during or at 30 min after pulse application at 37°C (open columns) and 40°C (filled columns). PC filter is a non-temperature-sensitive polycarbonate control barrier.

after electroporation is an important factor controlling the efficiency of delivery. Most of the transport, in fact, takes place during the prolonged recovery period after the pulse if the molecules to be transported remain in the donor chamber. Figure 3 shows the relative amount of Dextran 10kDa at various compartments and times after pulsing. Apparently, most of the transport occurred during the post-pulse incubation period of 30 minutes at 40°C. This knowledge forms the basis of improving our pulse protocol.

2. Optimization of delivery protocol for antigenic peptides: The K^b-binding OVA peptide, SIINFEKL, and the HLA-A2 binding HER-2/neu peptide, KIFGSLAFL, were synthesized and purified. In order to measure the quantity of peptides that can be delivered transdermally by electroporation, some of the OVA peptide was labeled with rhodamine. This peptide carries 1 net negative charge when the lysine is linked to a rhodamine. The transport across excised murine skin was measured using a Vertical Diffusion Holder as described above. The donor chamber contains the cathode while the receiver chamber contains the anode. The amount of peptide that could be delivered to and through the skin was about 1 µg/cm² after fifteen 1 msec pulses of 100V were applied at 1 Hz. An undetermined portion of
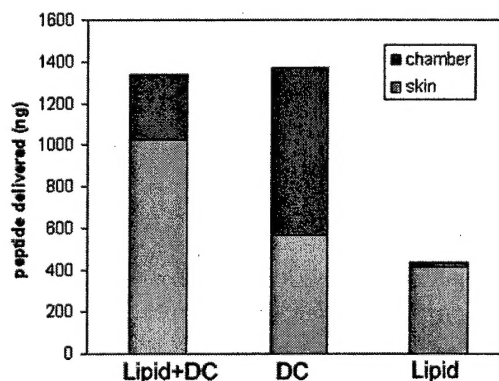

Figure 4. Transport of the SIINFEKL-rhodamine peptide to murine skin, in the presence or absence of anionic lipids or a 9VDC potential after fifteen 1 msec pulses of 100V were applied at 1 Hz.

the labeled peptide was visibly retained by the remaining hair even after clean shaving. Application of anionic lipids tended to encourage the peptide to remain in the skin rather than

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passing through to the receiving chamber (figure 4). Application of a 9VDC potential had the opposite effect. By increasing the local site temperature to 40°C and by leaving the peptide under the electrode for 30 minutes after pulse, the delivery amount can be increased by an order of magnitude.

3. Improving the design of transdermal delivery electrodes: In order to maximize the transdermal delivery of antigenic peptides to murine skin *in vivo*, special electrodes were designed and used. The initial electrode design was modified from a commercial Biopotential Skin Electrode E256A (In Vivo Metric, Ukiah, CA). The cavity in front of the AgCl electrode was filled with highly conductive Sigma Gel (Parker Laboratory, Orange, NJ). 20 µl of the peptide to be delivered was soaked onto a filter disc and placed in between the skin surface and the gel-filled electrode. The skin surface was cleanly shaved and locally wetted with conducting buffered solution (100 mM NaCl, 10 mM TRIS pH. 7.4) and anionic lipids when needed. This design was first tested *in vitro* with a Vertical Diffusion Holder, then *in vivo* with mice.

Because of the need to increase the delivery quantity, in a later design, the contact area on the skin was increased 5-fold, and the filter disc was replaced by a thin liquid chamber and sealed to the skin surface. The liquid chamber can hold 250 µl of concentrated peptide. Replacing the filter disc with liquid layer facilitated convection of the fluid during the diffusion period when most of the transport takes place.

4. Monitoring Langerhans cell migration resulting from electric pulse application: The migration of Langerhans cells (LC) away from the pulsed skin areas was monitored. LC migration can be initiated by topical application of allergens (1% of 2,4,6-trinitrochlorobenzene dissolved in paraffin oil). It is postulated that the migration can also be triggered by the stimulation of electric pulse application. LCs were recognized by MHC-class II labels in fluorescence microscopy, as described in Ostberg et al. (2000). The same pulse protocol (fifteen to sixty 1 msec pulses of 100V at 1 Hz), as used in transport measurements, was applied to the ears of Balb/c mice, using the modified AgCl skin electrode. The ears were removed and cultured for 6, 24 and 48 hrs as described in Ostberg et al. (2000). The number of LC migrated from or remained in the ears were counted. The increase of the former usually accompanied with a decrease of the latter. The number of LC migrated to the culture dishes were more accurately counted and presented in table 1.

Table 1. Migration of LC after Electropulse

Post-pulse time	No pulse	100 V, 15 pulses	100 V, 60 pulses
24 hrs	7,500	27,500	47,500
48 hrs	40,000	60,000	90,000

5. Measurement of CTL response after immunization of antigenic peptides: The HLA-A2 binding p369 peptide, KIFGSLAFL, is a weak antigen. The CTL response to this antigen can be detected only after *in vitro* T-cell expansion (Lustgarten et al., 1997). For preliminary evaluation, it was decided initially to compare the delivery of this peptide by intradermal (i.d.) injection and by electroporation. P369 was delivered to HLA-A2/K[b] transgenic mice as a vaccine, and the CTL response of the immunized mice was monitored. For each experiment, 4 mice received peptide delivery by transdermal electroporation. 100 µg of the peptide in 20 µl was soaked onto a filter disc and placed in between the skin surface and the gel-filled delivery electrode. The

reference electrode was of the same construction but was connected to the opposite polarity and with a buffer-filled filter disc instead of a peptide-filled one. The p369 peptide KIFGSLAFL has a net positive charge and was placed under the anode for delivery. The skin surface was locally wetted with conducting buffered solution (100 mM NaCl, 10 mM TRIS pH. 7.4) to ensure good electric contact. Mice were anesthetized before application of electric pulses. 20 pulses at 250V (125V per skin passage) and 1 msec duration were applied at 1 Hz. After pulsing, the peptide solution was left on the skin for 15-30 min to take advantage of post-pulse diffusion. As positive control, 2 mice were injected i.d. with the same amount of peptide together with Freund's complete adjuvant and a 20-mer peptide T-helper promoter. For fair comparison, same adjuvants were injected i.d. at the electroporation site after pulsing. Booster delivery was made after 14 days but with Freund's incomplete adjuvant. 2 untreated mice served as negative control. 14 days after booster delivery, mice were sacrificed, and spleenocytes were removed for CTL assay. The more sensitive ELISPOT assay (Manjili et al., 2002) was used instead of the originally proposed chromate release assay for CTL response. In brief, spleenocytes were dissociated and stimulated with 1 µg/ml of the peptide (or con-A for positive control) and cultured on 96-well filter plates for 20 hr. After repeated washing, the filter plates were labeled with biotinylated IFN-γ antibody. The plates were developed in alkaline phosphatase avidine-D and BCIP/NBT. The spots per well were counted under a microscope. Without *in vitro* T-cell expansion, mice immunized by i.d. injection of the peptide show very weak and inconsistent response, and those by electroporation have shown no detectable CTL response. In order to properly test the electroporation technique, we decided then to use the stronger antigenic OVA peptide, SIINFEKL, rather than pursuing *in vitro* T-cell expansion that may add a further variable step.

The OVA peptide is more water-soluble than p369, reaching a saturation concentration of 0.5 mg/ml. This is important because we need to maximize the diffusion gradient for increasing the amount of peptide to be transported. In a separate experiment, we found that applying electroporation in the presence of 3 mg/ml of sodium dodecylsulfate (SDS) solution increases transport by at least 5-fold, while SDS solution alone has no effect in enhancing permeability with 30 minutes of application. Furthermore, the OVA peptide can be dissolved in SDS solution up to 1.3 mg/ml. This was than used in a comparison experiment between i.d. injection and electroporation delivery. Since the OVA peptide is K^b –specific, C57BL/6 mice were used. The delivery protocol and assay methods were similar to that for the p369 peptide as described in the last paragraph.
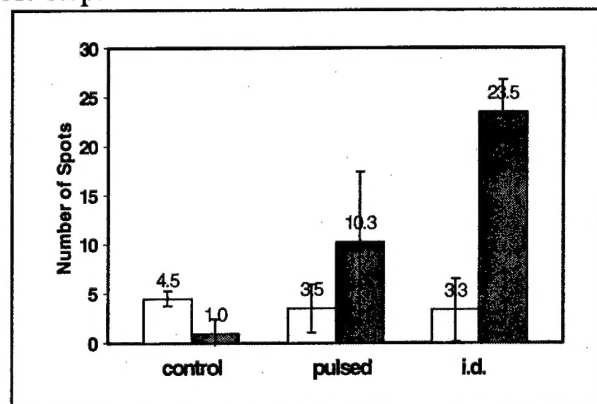

Figure 5. Number of spots due to antigen-specific response (filled bar) or non-specific response (open bar) from ELISPOT assay of CTL activity, after OVA peptide vaccination by electroporation (pulsed) or by i.d. injection. Error bars represent standard deviations.

Figure 5 shows the results from the ELISPOT assay. Although the antigen-specific CTL response by electroporation vaccination is not as high as that by i.d. injection, it has an above-background immune response. One reason for the lower response by electroporation vaccination is that the adjuvant injection site may not completely overlap the electroporation site. We are

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currently investigating the use of CpG-containing oligonucleotides (ODN) as adjuvant (Weeratna et al., 2000). The advantage of using ODN as adjuvant is that its smaller molecular size may enable it to be delivered simultaneously with the peptide vaccine by electroporation without separate injection. The results of this on-going study are not yet available.

KEY RESEARCH ACCOMPLISHMENTS:
- Increased the efficiency of molecular transport and extended the upper molecular weight limit of transdermal delivery by improving electroporation technology.
- Optimized conditions for delivering antigenic peptides to the skin by electroporation.
- Established that the electroporation process does initiate Langerhans cell migration.
- Improved electrode design for further enhancing peptide delivery.
- Proved that peptide vaccines can be delivered by transdermal electroporation, as judging by specific CTL activation assay.

REPORTABLE OUTCOMES:
1. Zhao, Y.L., Murthy, N.S., Sen, A. and Hui, S.W. Transdermal delivery of peptide vaccines by electroporation. (in preparation)

CONCLUSIONS:
Successful delivery of the HER-2/neu derived p369 peptide vaccine, KIFGSLAFL, to a large population of skin Langerhans cells (LC) by transdermal electroporation is expected to enhance the CTL response to breast cancer cells. Strategies have been developed to improve the electroporation protocol to enable the delivery of peptides. By using anionic lipids and detergents, the upper molecular weight limit of transdermal delivery by electroporation has been extended to beyond10,000 Da. This enables the delivery of antigenic peptides but not minigenes. With the new design of skin electrode, the transdermal flux of antigenic peptides (M.W.~9,000) was measured to be in the order of 10 μg/cm^2/min, when 1 msec pulses of 100V were applied to the skin at 1 Hz. Most of the transport was found to take place during the post-pulse diffusion period. An additional advantage of delivery by electroporation is that the electric pulse stimulates the migration of skin Langerhans cells. Antigen-specific CTL response in immunized mice was measured by ELISPOT of gamma-interferon production. Earlier attempts to generate CTL response to KIFGSLAFL by electroporation delivery were negative, due to weak immune response even to control injection. CTL response to electroporation delivery of the peptide vaccine SIINFEKL was positive, although it was not as effective as intradermal injection. We attribute the weaker response to the necessity to inject adjuvant separately. Effort to simultaneously deliver lower molecular weight CpG-containing oligonucleotide adjuvant by electroporation is underway. When the methodology is established, delivery of p369 or other HER-2/neu derived peptide vaccines will be re-examined.

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